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14. ABSTRACT

Mutations in the breast cancer susceptibility genes BRCA1 and BRCA2 are estimated to account for over 10% of the breast cancer burden in the US. The current standard of care for BRCA1 or BRCA2 mutation carriers is based on limited understanding of the effects of specific mutations, and thus treats all carriers as if they have the same relative risk for development of breast cancer. However, BRCA1 and BRCA2 tumors have very different phenotypes, and several regions within the genes that confer either reduced or enhanced risk for breast cancer have been identified. We proposed to create a model system of BRCA1 and BRCA2 heterozygous cell lines from women opting for prophylactic mastectomy, and have now collected a total of 16 samples. Epithelial cell isolation and immortalization is underway – we have one verified BRCA2 heterozygous cell line, two BRCA1 heterozygous cell lines in process, and normal control cell line immortalization is underway. Immediate future experiments will isolate mammary epithelial cells from all samples collected so far. These cell lines will comprise a model system that will be immediately transformative in the field of hereditary breast cancer biology.

15. SUBJECT TERMS
Mammary epithelial cells, human breast tissue, BRCA1, BRCA2

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Introduction

Mutations in the breast cancer susceptibility genes BRCA1 and BRCA2 are estimated to account for over 10% of the breast cancer burden in the US [1]. Prophylactic mastectomy, in concert with increased mammographic surveillance, remains the standard of care for BRCA1 or BRCA2 mutation carriers [1, 2]. To study the effects of heterozygosity at the BRCA1 or BRCA2 loci, immortalized mammary tumor cell lines have been created and challenged with DNA damaging agents and radiation [3-7]. These studies, as well as studies in non-mammary model systems, have shown that heterozygosity at either BRCA1 or BRCA2 decreases DNA repair efficiency and increases genomic instability, which presumably contributes to the development of further genetic mutations, driving tumor development [8, 9]. Most of these studies, however, involved a single cell line isolated from a single BRCA1 or BRCA2 mutation carrier, and comparisons across these studies are impossible due to the variety of immortalization techniques used and the diversity of techniques used to examine DNA damage repair. Therefore, there is a need for a consistent model system created from non-tumor cell lines isolated from multiple BRCA1 and BRCA2 individuals, generated with standard isolation and immortalization protocols. I have begun to make such a model system, to allow the effects of BRCA1 and BRCA2 mutations on normal mammary gland biology and carcinogenesis to be studied in a consistent and comprehensive manner. This model system will also allow us to study the differences between mutations in the BRCA1 or BRCA2 loci occuring at different regions of the gene, and to examine the impact of increased lifetime exposure to radiation, via increased mammographic screening. This information is critical to updating our current standards of care for BRCA1 and BRCA2 mutation carriers.

Report Body

Task 1: *Examine impact of BRCA genes on DNA damage and repair in existing BRCA1 and BRCA2 +/+ (wild-type) and -/- (null) cell lines. (Months 1-12)*

The work for this aim was mostly completed, as projected, during the first year of the grant. We have performed some additional analyses using bleomycin as the DNA damaging agent, now that our assay systems are optimized.

1.1: Establish the time course of double strand break (DSB) formation and repair in cells exposed to increasing doses of ionizing radiation or bleomycin.

Histone H2A.X phosphorylation at serine 139 is a known DNA damage indicator, and one that we have used previously (Annual Report, year 1, Figure 5). We performed an immunofluorescence assay for phosphor-histone H2A.X using bleomycin as a DNA damaging agent, according to the protocol detailed in the year 1 Annual Report. We opted to use the hTERT-HME cell line alone, due to the high cost of bleomycin as compared to ionizing radiation. Briefly, hTERT-HME cells were seeded onto chamber slides, and treated 24 hours later with indicated doses of bleomycin or water control. 48 and 72 hours later, cells were fixed with formaldehyde and permeabilized with ice-cold methanol, then incubated with an antibody

against histone H2A.X phosphorylated at serine 139. A secondary antibody conjugated to Alexa Fluor 488 dye was subsequently used, followed by DNA counterstaining with DAPI. Slides were viewed on a Zeiss Axioskop 2 microscope and images were captured with a Zeiss Axiocam digital camera. DNA double strand breaks are induced in hTERT-HME cells at a dose of bleomycin as low as 10ng/mL at 48 hours, but staining levels are lower at 72 hours, indicating a resolution of the DNA strand break (Figure 1). At bleomycin doses of 10μg/mL or higher, DNA double strand breaks persist at 72 hours, indicating either an inability to repair DNA damage, or a lag in DNA damage repair. These data correlate well with our previous data; hTERT-HME cells are growth inhibited by bleomycin at doses of 1μg/mL or higher, which persists at 96 hours (Annual Report, year 1, Figure 1).

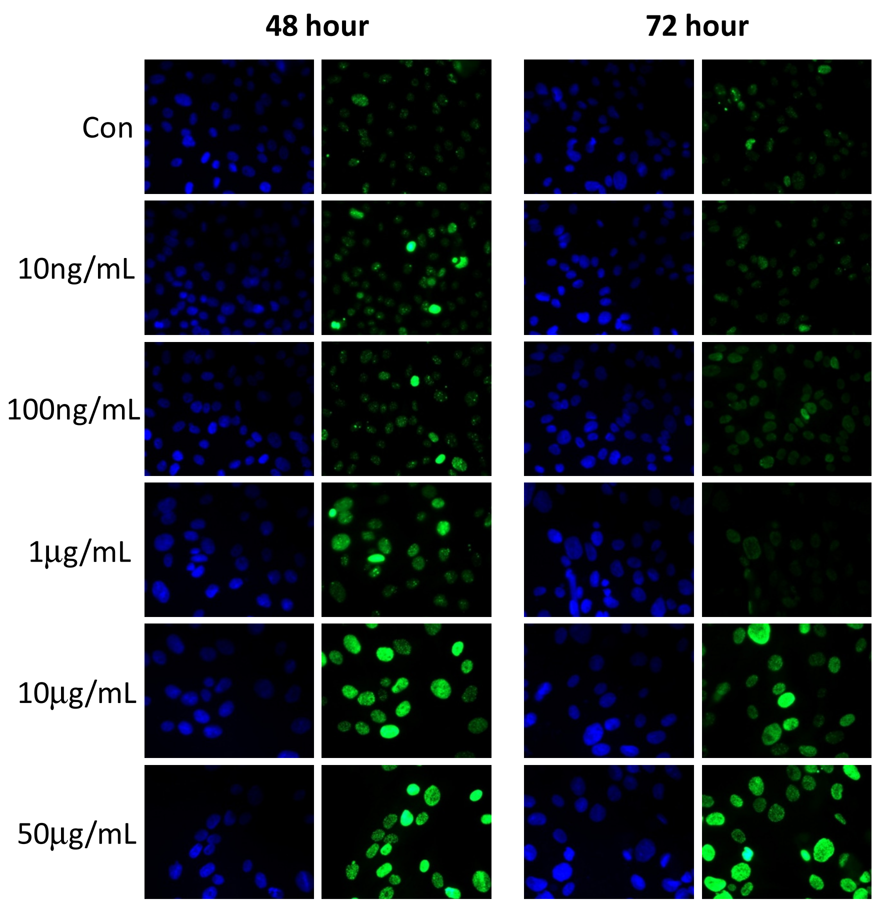


Figure 1 – DNA double strand breaks are induced in hTERT-HME cells following treatment with bleomycin, and persist for up to 72 hours. Immunofluorescence images of anti-phospho histone H2A.X (Ser139) staining 48 and 72 hours post-treatment with indicated levels of bleomycin or vehicle control.

1.2: Examine transformation following DNA damage in cell lines.

We are still working towards optimizing standard soft agar anchorage-independent growth assays, given the high cost of the kit we were using formerly. We will be performing these assays in our new BRCA +/- cell lines (described in Aims 2 and 3) soon.

1.3: Measure BRCA1 and/or BRCA2 protein and mRNA expression, and determine BRCA1 and/or BRCA2 protein localization during DSB formation and repair.

In our last annual report, we showed a western blot for the BRCA1 and BRCA2 proteinsfour hours post-treatment in each of the five existing cell models (Annual Report, year 1, Figure 11). We wanted to examine whether BRCA1 or BRCA2 protein levels were changing over time following treatment with ionizing radiation or bleomycin treatment, but had a great deal of difficulty resolving either BRCA1 or BRCA2 proteins at longer time points (24-72 hours) (Figure 2). Up to this point, we had been using Tris-Glycine gels for all of our western blotting, but decided to try using Tris-Acetate gradient gels; Tris-Acetate gels are known to be better for resolving large proteins, and gradient gels would allow us to visualize other proteins along with the BRCAs. As shown in Figure 3, the Tris-Acetate gels did resolve the BRCA1 and BRCA2 proteins better than Tris-Glycine gels after 48 hours of treatment, however the results vary between the two independent replicates we performed. We have switched to using these Tris-Acetate gels for all further western blot experiments, and are continuing to optimize the assay conditions.

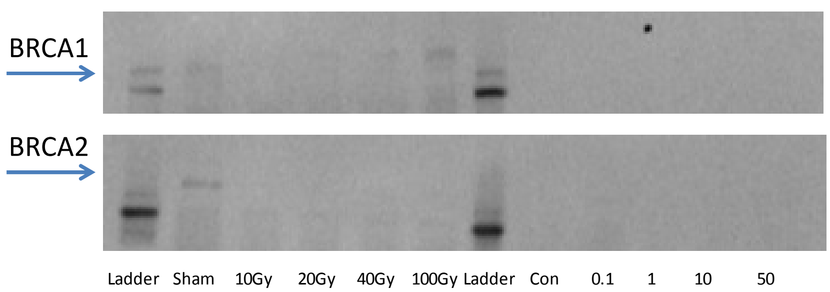


Figure 2 – BRCA1 and BRCA2 protein expression is not easily resolved in hTERT-HME cells following 48 hours treatment with either ionizing radiation or bleomycin. Western blot analysis 48 hours post-treatment with ionizing radiation or bleomycin at indicated doses. Loading was controlled for via actin staining (not shown). The top band in the “Ladder” lane is approximately 200kDa.

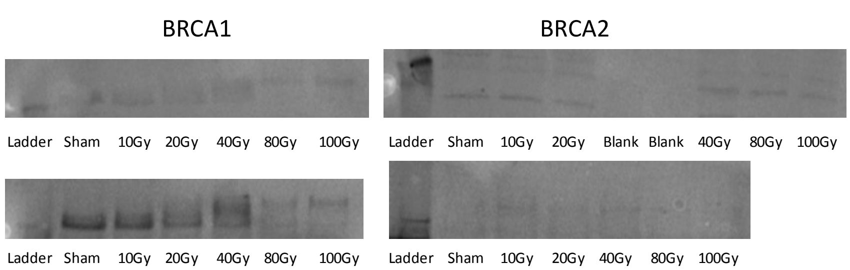


Figure 3 – Tris-Acetate gels resolve the BRCA1 and BRCA2 proteins better than Tris-Glycine gels for long-term study. Western blot analysis in hTERT-HME cells 48 hours post-treatment with ionizing radiation at indicated doses. Loading was controlled for via actin staining (not shown).

To examine BRCA1 and BRCA2 protein localization during DNA double strand break repair, we performed immunofluorescence assays in hTERT-HME cells 24 and 48 hours post-treatment with bleomycin. We chose to focus on the hTERT-HME cells in this experiment, as the other 4 test cell lines have compromised DNA DSB repair responses, and vary in their BRCA1 and BRCA2 expression. We also examined the localization of GADD45, which is upregulated following DNA damage and activates the p38/JNK pathway to induce growth arrest and/or apoptosis. Immunofluorescence was performed as detailed above, with DAPI used as a counterstain (data not shown). A no-primary control was also performed at each timepoint with each treatment, to ensure that all green staining seen was antibody-specific and not due to non-specific background staining (data not shown). While it is difficult to see at this resolution, puncta of green are seen in cells treated with 10ng/mL bleomycin or higher in each of the three antibody conditions; this indicates that DNA damage repair proteins, such as BRCA1, BRCA2, and GADD45, are localizing on the DNA at sites of double strand breaks (Figure 4). These puncta are located in the nucleus (indicated by overlap with DAPI staining, data not shown) and persist at 48 hours under all conditions, showing that DNA damage repair is ongoing at that time; this correlates well with our H2A.X staining in hTERT-HME cells treated with bleomycin (Figure 1). BRCA1 and BRCA2 appear to be localized mainly in the nucleus, even under control conditions, while GADD45 protein is localized throughout the cell, but concentrated in the nucleus following DNA damage. The amount of GADD45 protein also appears to increase following DNA damage. BRCA2 staining was weaker than BRCA1 or GADD45, but still shows the puncta following bleomycin treatment (Figure 4). Overall, these data indicate that BRCA1 and BRCA2 are correctly localizing in hTERT-HME normal mammary epithelial cells following DNA double strand breaks.

Task 2: *Establish 20 immortalized BRCA1 and BRCA2 heterozygous mammary epithelial cell lines (MECs). (Months 1-36)*

In the past year, we have collected an additional 14 patient samples, for a grant-to-date total of 16. We received our IRB protocol yearly continuation with no major issues or problems noted. We anticipate a greater volume of tissue collection in the third year of the grant, as Dr. Ashikari (our collaborator) has recently started operating out of a second, larger hospital, allowing for more surgeries to be performed each month.

Our IRB protocol and grant Human Subjects documentation each required us to name and number the patient samples (and resulting cell lines) in a way that was anonymous. We have settled on the acronym “MCK” as a base name for the cell lines generated in this study – each letter stands for the first initial of a woman in Dr. Keith’s family who died from BRCA2-related cancers. The numbering system, while anonymous, gives an indication as to whether BRCA1 or BRCA2 is mutated in that particular sample; a “1” as the first number indicates a BRCA1 mutation, while a “2” indicates a BRCA2 mutation.

Table 1 shows the name, mutation, and age at time of surgery for each of the patient samples collected to date. We currently have a 5:3 ratio of BRCA1:BRCA2 (10 BRCA1 samples and 6

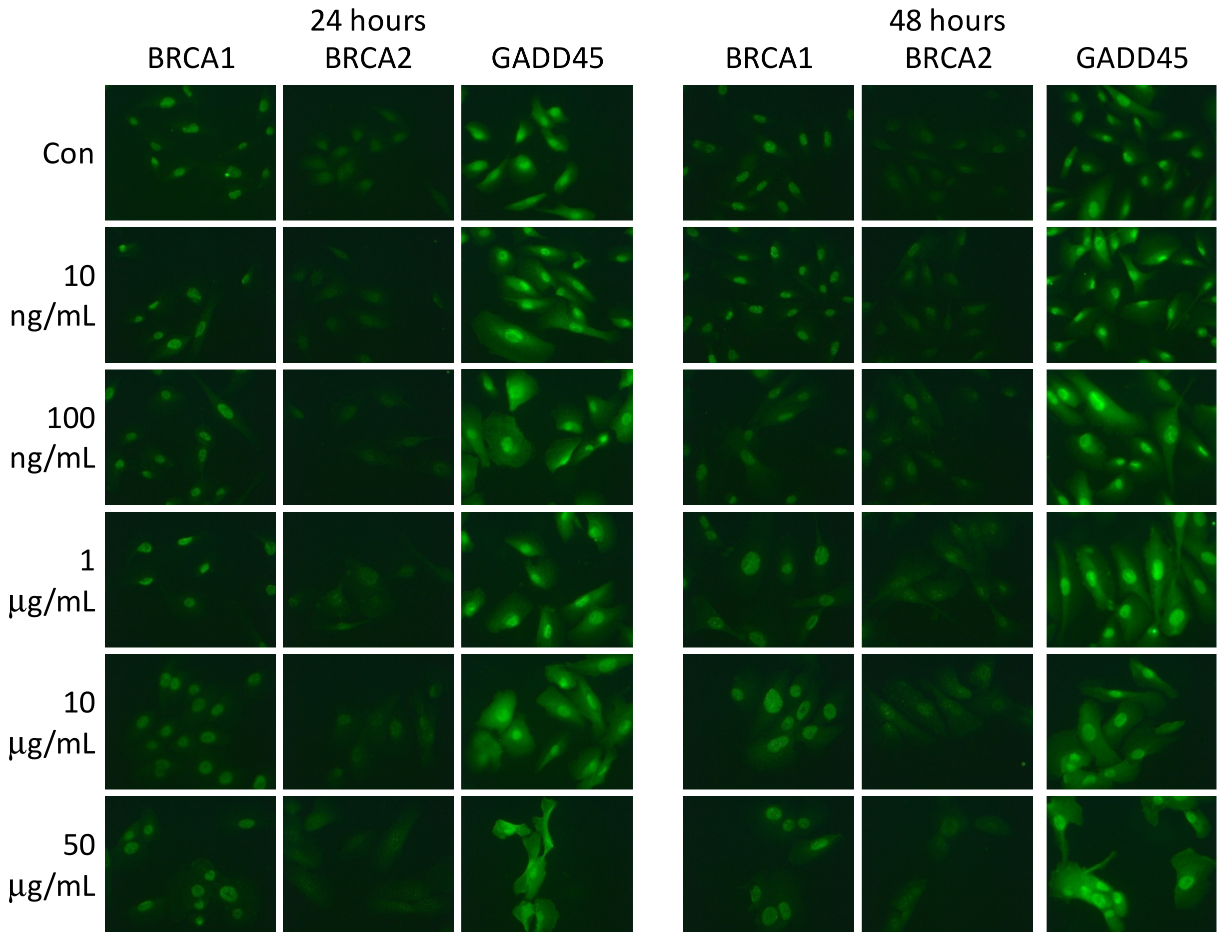


Figure 4 – DNA double strand break repair proteins localize to the nucleus in discrete foci following DNA damage in hTERT-HME cells. Immunoflouorescence images of anti-BRCA1, anti-BRCA2, and anti-GADD45 staining 24 and 48 hours post-treatment with indicated levels of bleomycin or vehicle control.

BRCA2). Half (five) of the BRCA1 samples contain the 185delAG Ashkenazi mutation – this is the most common BRCA mutation, so it is unsurprising that we have collected multiple samples containing it. One-third (two) of the BRCA2 samples contain the 6174delT Ashkenazi mutation, which is the most common BRCA2 mutation. Our patient ages range from 25-68, which is within the range we anticipated; only two patients to date have been above the age of 60 (Table 1). The “Result” column in Table 1 indicates what the consequences of each of the mutations are on the resulting protein product. In some cases, specific truncation sites following frameshift mutations are known, while in others, the outcome is less clearly defined. The specific truncation sites were taken from the Breast Cancer Information Core (BIC) database, maintained by NHGRI (http://research.nhgri.nih.gov/bic/).

As we proposed in last year’s annual report, we have adjusted our tissue digestion protocol, and are meeting with much better results. MCK11 and MCK21 were digested according to the original protocol (Annual Report, year 1, page 13-14), while all other MCK samples were

digested according to the new protocol, detailed below. In brief, fatty tissue was removed with

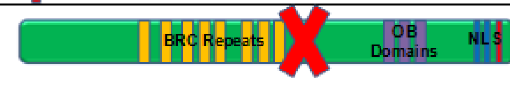
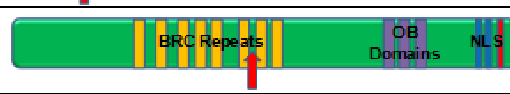
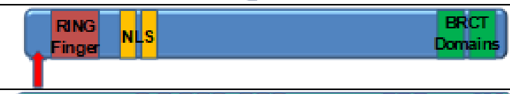
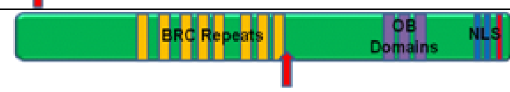
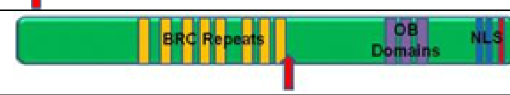
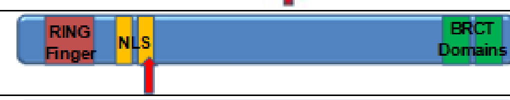
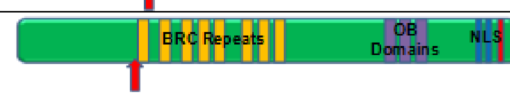
Name	Age	Mutation	Location	Result
MCK11	37	BRCA1 185 del AG		Truncation - stop codon 39
MCK21	63	BRCA2 del exon 12		?
MCK22	51	BRCA2 K1374E		Missense
MCK12	44	BRCA1 5382 ins C		Truncation - stop codon 1829
MCK13	34	BRCA1 288 del A		Frameshift
MCK23	46	BRCA2 5057 del TG		Truncation - stop codon 1613
MCK14	25	BRCA1 185 del AG		Truncation - stop codon 39
MCK24	68	BRCA2 6174 del T		Truncation - stop codon 20036
MCK15	25	BRCA1 185 del AG		Truncation - stop codon 39
MCK25	38	BRCA2 6174 del T		Truncation - stop codon 20036
MCK16	27	BRCA1 1629 del C		Truncation - stop codon 531
MCK26	44	BRCA2 1377 ins XG		Nonsense
MCK17	55	BRCA1 R1495M		Missense
MCK18	45	BRCA1 del exons 18-19		?
MCK19	30	BRCA1 185 del AG		Truncation - stop codon 39
MCK101	27	BRCA1 185 del AG		Truncation - stop codon 39

Table 1 – Basic characteristics of the MCK patient samples collected to date. "Age" refers to patient age at time of surgery. "Location" is a graphical representation of the approximate location of the mutation in that MCK line in reference to the rest of the protein; BRCA1 is shown in blue and BRCA2 in green. "Result" indicates what the expressed protein product of each mutation would consist of.

scalpels, and the remaining tissue was lacerated and minced with opposing scalpels. Minced pieces were placed into a 50mL Erlenmeyer flask, containing 10mL digestion medium (Ham's F-12 containing insulin, penicillin, streptomycin, polymyxin B, Fungizone, 10% fetal bovine serum, collagenase, and hyaluronidase) for one hour. At the end of the hour, the digestion media was removed from the flask, and 1mL seeded per well in 6-well plates. 10mL of fresh digestion medium was then added to the flask, and the tissue digested for another hour. This hourly process was repeated for as long as necessary to complete digestion of the tissue samples (10-12 hours normally).

This new protocol provides for a greater sample yield, as outgrowth occurs from the cells seeded at almost every hour timepoint, and also gives us a greater chance to isolate epithelial cell colonies from each tissue sample. We have frozen aliquots of each MCK sample at each hour timepoint at -80C for future use.

Our initial intent was to immortalize the MCK cell lines using the pBabe-puro-hTERT vector as previously discussed. We tried first with MCK22 and MCK12, as these were the first cell lines that showed good epithelial outgrowth in the primary digested cultures. We infected cultures of grossly epithelial-looking cells with frozen retroviral infection stocks, and selected with varying concentrations of puromycin for 5 days. At the end of the selection phase, cell cultures were rescued with M87A growth medium in the absence of selection. At first, we appeared to have stably selected cell lines (see Figures 5-7), but we were never able to achieve passage numbers greater than 5 in either the MCK22-hTERT or MCK12-hTERT stable cell lines.

Upon further reading and discussion with colleagues, we began to suspect that we would have to knock out either p53 or pRb signaling, in addition to stably expressing hTERT, in order to achieve full immortalization [10]. At first, we left the morphologically senescent MCK22 and MCK12 cultures to grow, changing the medium every 2-3 days, as spontaneous reversal of agonescence is the commonly used method to date. One month elapsed with no change in cell growth or morphology in either cell culture. We then decided to manually alter pRb signaling; in most epithelial cells undergoing natural agonescence, pRb signaling is blocked by alterations in p16 protein production, usually via hypermethylation of the p16 gene promoter. We felt that a short hairpin RNA against p16 would achieve this same effect, while leaving p53 signaling intact.

We obtained the pLenti X2 Blast/shp16 (plasmid ID# 22261) from Addgene, and cloned out the p16 short hairpin RNA sequence. We added this fragment to our pBabe-puro-hTERT stable retroviral expression vector via site-directed cloning. Sequencing of the plasmid confirmed that the p16 short hairpin sequence was intact, and not interfering with other important elements in the pBabe-puro-hTERT vector. We have named this new vector pBabe-hTERT-shp16, and are now using it to immortalize the MCK cell lines.

The MCK25 cell line was the first sample set to be infected with pBabe-hTERT-shp16. These stably immortalized cell cultures are now at passage 8 post-infection, and show no signs of senescence. The MCK26 and MCK17 cell samples yielded mostly fibroblastic cells post-digestion, and the MCK18 cell sample did not show any growth in culture following digestion. Currently, the MCK19 and MCK101 cell samples are post-selection, and are in the rescue

phase of immortalization. Each shows grossly epithelial cell clusters, which will be cloned and pooled, to create the immortalized line. We have also tried thawing prior freezes (MCK11-MCK24 on the chart) and immortalizing them, but are having difficulty obtaining epithelial colonies from these frozen stocks. If we cannot obtain epithelial cell colonies, we will immortalize and select fibroblastic cultures for use in cell-cell signaling experiments. We are also in the process of creating a stable control cell line. Our IRB protocol does not allow us to collect tissue samples from women who do not have BRCA mutations, so we have ordered a vial of primary normal human mammary epithelial cells (PHMECs) from Invitrogen, and will infect them with the pBabe-hTERT-shp16 vector.

Task 3: *Characterize BRCA1 and BRCA2 heterozygote MEC model system, and examine differential effects of BRCA1 or BRCA2 mutation position on response to DNA damage and/or chemopreventive agents. (Months 6-36)*

Following infection with the pBabe-puro-hTERT retroviral expression vector, both the MCK22-hTERT and MCK12-hTERT cell lines appeared to be stably immortalized. We initially wanted to assess whether BRCA1 or BRCA2 haploinsufficiency would affect the response of these mammary epithelial cell lines to ionizing radiation or bleomycin. Cells were seeded into five 24-well plates per cell line, and treated 24 horus later with the indicated dose of ionizing radiation or bleomycin. One plate per cell line was fixed with 1% glutaraldehyde each day, beginning with the day of treatment (day 0) and ending 96 hours post-treatment (day 4). At the end of the experiment, all plates were stained with crystal violet dye, and absorbance read at 590nm, which is equivalent to cell number. In the graphs below, the hTERT-HME testing was done in parallel with the MCK cell lines, while the HCC1937 and Capan-1 data has been previously presented (in Annual Report 1), and is shown here for comparison.

As shown in Figure 5, hTERT-HME wild-type cells are growth inhibited by irradiation at levels as low as 10Gy, but rebound and continue growth as early as 48 hours post-irradiation (Figure 5), even at doses as high as 100Gy (data not shown). Both HCC1937 and Capan-1 BRCA1/BRCA2 null cell lines are growth inhibited by doses as low as 10Gy but cannot rebound and grow, even at 72 hours post-dose (Figure 5). MCK22-hTERT BRCA2 heterozygous and MCK12-hTERT BRCA1 heterozygous precancerous cells are slightly growth inhibited by irradiation, and show a rebound with continued growth. The growth inhibition is not as strong as that seen in hTERT-HME cells, which may be due to the relatively slow growth rate of the MCK3 and MCK4-hT cells. This indicates that ionizing radiation induces DNA double strand breaks in all cell lines, which hTERT-HME, MCK3, and MCK4-hT cells are able to repair, while BRCA1 and BRCA2 null cell lines cannot.

Figure 6 shows that hTERT-HME cells are growth inhibited by bleomycin at levels of 10ng/mL or greater, with no apparent loss of cell number, even at 10μg/mL (Figure 6). In contrast, both BRCA1 and BRCA2 null cell lines show decreases in cell density following bleomycin treatment, at effective levels which vary from cell line to cell line. MCK3 and MCK4-hT heterozygous precancerous cells show a growth profile similar to that of the hTERT-HME cell line, with sensitivity at 10ng/mL and no evidence of cell death (Figure 2). This suggests that, like ionizing radiation, bleomycin induces DNA double strand breaks in these cell lines, which causes

hTERT-HME and MCK cells, which express the BRCA proteins, to growth arrest and BRCA1 and BRCA2 null cells to undergo death.

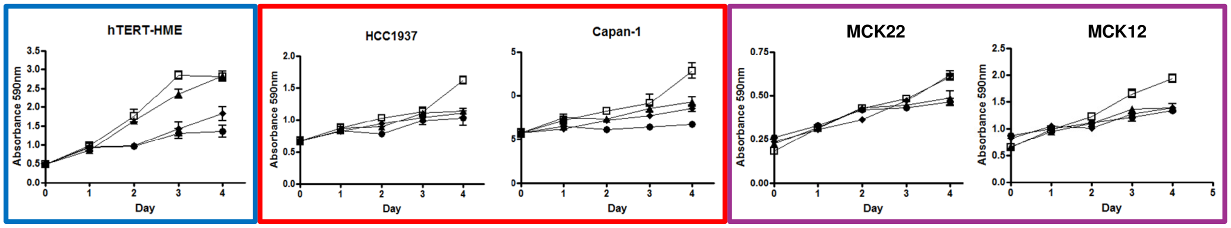


Figure 5: Ionizing radiation causes growth arrest in all cell lines, but only hTERT-HME cells can fully recover and grow. Crystal violet timecourse from 24 to 96 hours post-treatment with indicated doses of ionizing radiation: Sham (open squares), 10Gy (filled triangles), 20Gy (filled diamonds), or 40Gy (filled circles). Each data point represents mean ± SEM of three independent replicates.

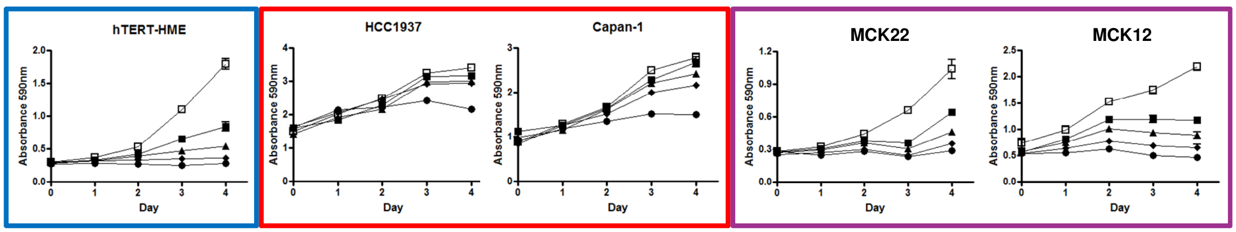


Figure 6 - : Bleomycin causes growth arrest in wild-type and BRCA1 heterozygous cells, and decreases cell number in BRCA null cells. Crystal violet timecourse from 24 to 96 hours post-treatment with indicated doses of bleomycin: Control (open squares), 10ng/mL (filled squares), 100ng/mL (filled triangles), 1mg/mL (filled diamonds), or 10mg/mL (filled circles). Each data point represents mean ± SEM of three independent replicates.

With evidence that the DNA was being damaged after treatment with bleomycin and ionizing radiation, we wanted to determine whether the MCK cell lines mount an appropriate DNA damage response. We performed western blotting for the p53 protein, which is known to be involved in DNA repair, and correlated the changes in p53 protein expression with H2A.X, as a measure of DNA damage. The hTERT-HME and MCK22 blots were performed simultaneously, while the HCC1937 and Capan-1 blots have been previously presented (Annual Report 1), and are shown for comparison. Figure 7 shows that in BRCA1 and BRCA2 wild-type hTERT-HME cells, bleomycin at doses of 1 μg/mL and above induce DNA double strand breaks (as measured by H2A.X) and cause an increase in the levels of cellular p53 protein. This suggests that the hTERT-HME cells detect DNA damage and mount an appropriate repair response. In both the HCC1937 BRCA1 null and Capan-1 BRCA2 null cell lines, the DNA is damaged by bleomycin at 10 and 50 μg/mL doses, but since both cell lines harbor p53 inactivating mutations (truncated in HCC1937 and a point mutation in Capan-1), the levels of p53 mutant protein are not increased following DNA damage. This suggests that these cells are unable to mount a DNA repair response following DNA damage. In MCK22 heterozygous precancerous cells, DNA DSBs are induced by bleomycin concentrations of 1 μg/mL or higher, and p53 protein is

upregulated, indicating that these BRCA1 heterozygous cells are able to detect and repair DNA DSBs.

Following our discovery that the MCK12-hTERT and MCK22-hTERT stable cell lines were not fully immortalized, we created and began using the pBabe-hTERT-shp16 vector for stable cell infection and immortalization. To date, only the MCK25-hTERT-shp16 cell line has been stably transfected, selected, and recovered fully enough to use for experiments. The MCK19 and MCK101 cell lines will be ready for use within the next several weeks.

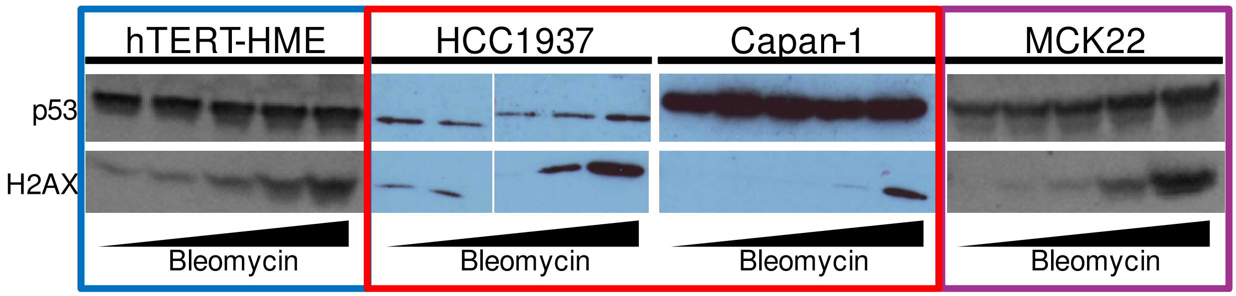


Figure 7 – p53 protein levels are increased in hTERT-HME and MCK22 cells following DNA double strand break induction. Western blot analysis 24 hours post-treatment with increasing doses of bleomycin, from control to 50µg/mL. Loading was controlled for via Ponceau staining of blots (not shown).

Initially, we had only enough MCK25-hTERT-shp16 stable cells to perform single-plate crystal violet experiments. To maximize data generation, we treated one plate with increasing doses of ionizing radiation, and one with increasing doses of bleomycin. Figure 8 shows the results of this initial experiment. We first noticed the low absorbance readings, indicating an overall low level of cell growth – this correlates with what we've noticed as the cells grow. They are not senescent, but seem to grow much more slowly than the hTERT-HME cells, which corresponds to our observations in the MCK12 and MCK22 cell lines. However, even with slow cell growth, the MCK25-hTERT-shp16 cell line is growth inhibited by ionizing radiation and bleomycin, at doses with also cause growth arrest in hTERT-HME cells (Figure 8).

We wanted to examine the timecourse of this growth arrest, and determine whether it followed a similar course as that previously seen in hTERT-HME cells. Due to the slow growth of these cells, we chose to focus on bleomycin treatment in order to maximize the number of doses over time, rather than adding in ionizing radiation as well. We treated the MCK25-hTERT-shp16 cells with increasing doses of bleomycin (or control) for 0-5 days, fixing a plate each day. Growth inhibition by doses of bleomycin as low as 10ng/mL are clearly visible at 48 hours, and persist through 96 hours (Figure 9). This may suggest an overall decrease in the ability of the MCK25-hTERT-shp16 cell s to repair DNA double strand breaks and continue with the cell cycle. At 50µg/mL, bleomycin causes an overall decrease in cell number, which suggests cell death, although further studies are needed to confirm this finding. In contrast, hTERT-HME cells (shown in Figure 9 for comparison purposes) are resistant to doses of bleomycin below 1ug/mL, and show no decrease in cell number, even at the highest dose.

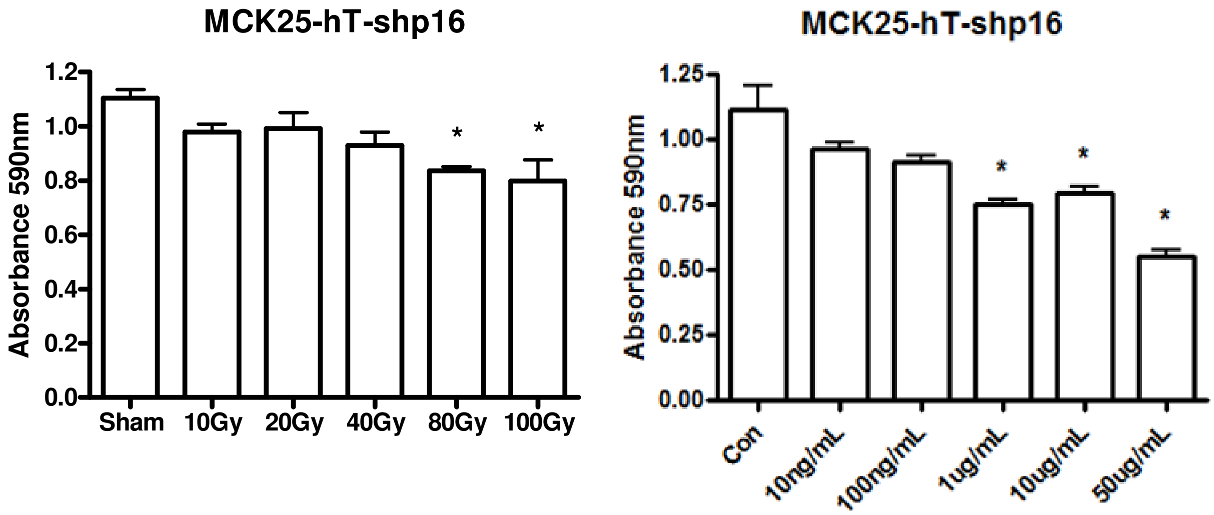


Figure 8 – Ionizing radiation and bleomycin cause growth arrest in MCK25-hTERT-shp16 immortalized BRCA2 +/- mammary epithelial cells. Crystal violet growth assay at 96 hours post-treatment with indicated doses of bleomycin or ionizing radiation. Each bar represents mean ± SEM of four replicates.

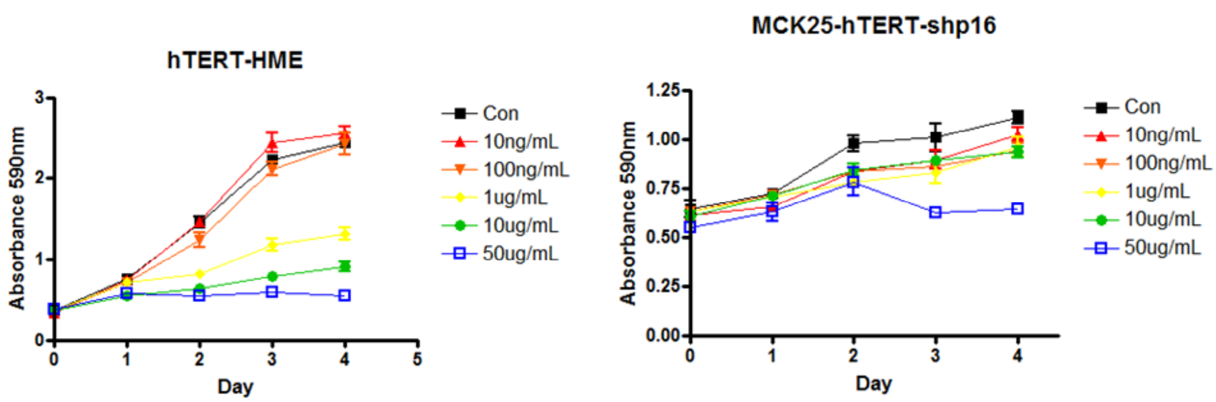


Figure 9 – MCK25-hTERT-shp16 cells are growth inhibited by bleomycin, and show slight decreases in cell number at high doses. Crystal violet time course from 24-96 hours post-treatment with indicated doses of bleomycin. Each data point represents mean ± SEM of four replicates. hTERT-HME graph shown for comparison only, and has been previously presented (Annual Report, Year 1, Figure 1).

Key Research Accomplishments

- Visualized DNA damage following bleomycin treatment in a quantifiable and easy-to-perform way (immunoflourescence)
- Optimized western blot protocol to allow for easier resolution of large proteins using Tris-Acetate gels
- Successfully resolved both BRCA1 and BRCA2 proteins via western blot analysis
- Determined DNA repair protein (BRCA1, BRCA2, and GADD45) localization in hTERT-HME cells following DNA double strand damage
- Collected 16 human mammary tissue samples to date
- Isolated and froze hourly digestion samples of mammary epithelial cells from each of the 16 tissue samples collected
- Created pBabe-hTERT-shp16 retroviral expression vector for one-step immortalization of mammary epithelial cells
- Immortalized the MCK25 cell line with the pBabe-hTERT-shp16 retroviral expression vector
- Determined that ionizing radiation induces DNA double strand breaks and growth arrest in BRCA +/- cells
- Determined that bleomycin induces DNA double strand breaks, growth arrest, and possibly cell death in BRCA +/- cells

Reportable Outcomes

An abstract has been submitted for presentation as a poster at the American Society of Human Genetics (ASHG) annual meeting, November 6-10 2012, in San Francisco, California. This abstract, “Modeling Carcinogenesis in BRCA1 and BRCA2 Heterozygous Mammary Epithelial Cells” details our progress on the work in Aims 2 and 3, and is included as Appendix 1.

To date, 16 patient samples have been collected, with a 10:6 ratio of BRCA1:BRCA2 mutations. Flash-frozen tissue samples and formaldehyde-fixed paraffin-embedded (FFPE) tissue blocks have been made from some of the tissue from each patient sample. The remaining tissue was used to isolate individual primary mammary epithelial cells, as detailed in the Report Body

above. These samples comprise a BRCA-mutation-focused research bank, and we will continue to add to the patient count and sample collection over the course of year 3 of the grant.

Using the data presented in last year's annual report, we have submitted applications for continuing funding for this project. An application titled "Modeling Carcinogenesis in BRCA1 and BRCA2 Heterozygous Mammary Epithelial Cells" was submitted to the New York State Department of Health Peter T. Rowley Breast Cancer Scientific Research Program, and would provide 2 more years of research funding, at $150,000 yearly. An R03 application titled "Modeling Dietary Chemoprevention in BRCA1/2 Heterozygous Mammary Epithelial Cells" was submitted to the National Institutes of Health (NIH) Cancer Prevention Research Small Grant Program, and would provide $50,000 in funding for two years. We are awaiting funding decisions for both of these grant opportunities.

Dr. Keith has begun applying for research faculty and teaching positions, to further her career beyond the postdoctoral stage. She has submitted applications to Skidmore College (Saratoga Springs, NY), Siena College (Latham, NY), The College of Saint Rose (Albany, NY), The State University of New York at Albany, and Berkshire Community College (Pittsfield, MA). Dr. Keith will continue to submit applications for research and/or teaching faculty positions over the course of the third year of the grant.

Conclusion

In the second year of our grant, we have successfully collected 16 BRCA1/2 heterozygous mammary tissue samples, and have begun isolating and immortalizing mammary epithelial cell lines which we have named the MCK cell lines. We completed the work proposed in Aim 1, and have now set all required experimental parameters; this will allow us to very quickly generate data with the MCK immortalized cell lines. Our initial studies with the MCK-hTERT-shp16 immortalized cell line, while limited in scope, have indicated that BRCA heterozygous cell lines are able to recognize DNA double strand breaks, and begin the DNA DSB repair response. This response seems blunted, however, when compared to immortalized normal mammary epithelial cells. We also have preliminary evidence that bleomycin is causing cell death in the MCK-hTERT-shp16 cell line, which is not seen in the hTERT-HME normal control, however further testing is required before any full conclusions can be drawn.

The most important advances we made this year were in collecting 14 additional patient samples (for a total of 16), and optimizing our immortalization protocol. One immortalized BRCA2 +/- cell line (MCK25) will soon be joined by several BRCA1 +/- cell lines, and a control wild-type cell line. Since year one was spent optimizing protocols for the majority of the experiments we proposed, we will be able to quickly generate data with each new immortalized cell line, and hope to generate at least 2 publications in year 3 of the grant. Once the cell lines are immortalized and characterized, we will work to distribute them to as many researchers as possible, to facilitate research on mammary cell transformation and tumor initiation.

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Appendices

Appendix 1: American Society of Human Genetics 2012 Meeting Abstract

Modeling Carcinogenesis in BRCA1 and BRCA2 Heterozygous Mammary Epithelial Cells

Mutations in the breast cancer susceptibility genes BRCA1 and BRCA2 account for half of all hereditary breast cancers, and are estimated to account for over 10% of the breast cancer burden in the US. The current standard of care for BRCA1 or BRCA2 mutation carriers is based on limited understanding of specific mutations, and thus treats all carriers as if they have the same relative risk for development of breast cancer. Several lines of evidence suggest that this is not true: BRCA1 and BRCA2 tumors have very different phenotypes, and specific mutations grant certain differential risk profiles for lifetime cancer incidence. Thus, we have created a novel model system of BRCA1 and BRCA2 heterozygous mammary epithelial cell lines, called MCK cells, from women opting for prophylactic mastectomy. These lines have been isolated from pre-neoplastic breast tissue that is not yet transformed. To date, we have collected 12

breast samples, with equal distribution between BRCA1 and BRCA2; this is the only model system which allows for comparisons between mutation sites within the BRCA genes.

The MCK cell lines are being immortalized for long-term use in cell culture. The effects of BRCA1 or BRCA2 mutation and the impact of different mutations within each gene on DNA repair and transformation will be assessed using these immortalized lines. Ionizing radiation is used as a DNA damaging agent, in order to mimic the effects of increased mammographic screening on BRCA mutation carriers. We will also examine the impacts of common dietary chemopreventive agents, such as vitamin D and EGCG, on growth and transformation in the MCK cell lines.

Our MCK panel of mammary epithelial cell lines will fill an immediate need in hereditary cancer research. The ability to compare and contrast mutations in differing sites within each gene will allow us to make more focused recommendations to BRCA mutation carriers, depending on the location of their particular mutation. In this way, we hope to “personalize” the management and treatment of BRCA1 and BRCA2 mutations, to enhance the quality of life of women who harbor deleterious mutations.

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